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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Beverly L. Davidson et al. Art Unit : 1648
Serial No. : 09/521,524 Examiner : Shanon A. Foley
Filed : March 8, 2000
Title : RAPID GENERATION OF RECOMBINANT ADENOVIRAL VECTORS

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF KEVIN CLARK UNDER 37 C.F.R. § 1.132

I, Kevin Clark, hereby declare as follows:

(1) That I am employed in the Library Department in the Boston office of Fish & Richardson P.C.

(2) That I contacted the publications department at Johns Hopkins University Press regarding the publication and mailing dates of the April 1999 issue of *Molecular Medicine*. They informed me that because they no longer publish *Molecular Medicine*, they were unable to provide me with any information. Johns Hopkins University Press referred me to North Shore Long Island Jewish Research Institute, the current publisher of the journal.

(3) That I attempted to contact Picower Institute Press and Springer Verlag, which apparently published the April 1999 issue of *Molecular Medicine*. As evidence of this, see the attached copy of the title page from that issue. I was unable to speak to anyone at Picower Institute Press, but I was able to get through to Springer Verlag. Springer Verlag informed me that because they no longer publish *Molecular Medicine*, they were unable to provide me with any information about the publication or mailing dates.

Applicant : Beverly L. Davidson et al.
Serial No. : 09/521,524
Filed : March 8, 2000
Page : 2 of 2

Attorney's Docket No.: 17023-005001 / 00015

(4) That I spoke with Octavia Davis at North Shore-Long Island Jewish Research Institute. Ms. Davis informed me that since North Shore did not publish *Molecular Medicine* in 1999, she was unable to provide me with the date the article was first made available to the public or the date it first appeared on-line.

(5) That the Library Department at Fish & Richardson P.C. was able to obtain a copy of the cover page and the inside cover page of the April 1999 issue of *Molecular Medicine* from the Countway Library of Medicine at Harvard University. The inside cover page was date stamped by the Countway Library. The date on the inside cover page is June 15, 1999.

(6) I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

April 20, 2004

Date

Kevin Clark
Kevin Clark

Molecular Medicine

OFFICIAL JOURNAL OF THE MOLECULAR MEDICINE SOCIETY

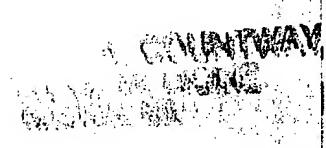
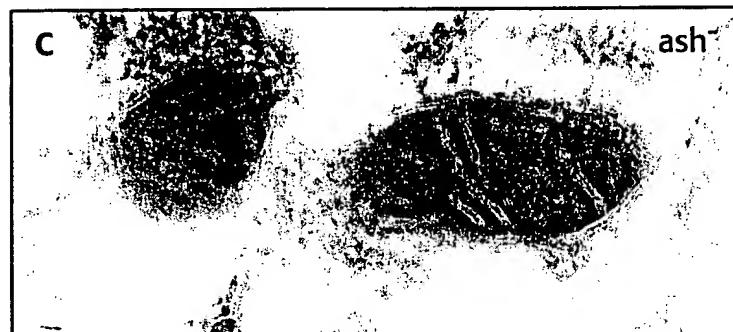
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EDITORS

EDITOR-IN-CHIEF

David Weatherall, FRS
Institute of Molecular Medicine
John Radcliffe Hospital
Oxford, United Kingdom

MANAGING EDITOR

Yvonne Cole, PhD
Picower Institute for Medical Research
350 Community Drive
Manhasset, NY 11030

CONTRIBUTING EDITORS

Frederick W. Alt, PhD

Children's Hospital
Harvard Medical School
Boston, MA

K. Frank Austen, MD

Brigham and Women's Hospital
Harvard Medical School
Boston, MA

Ernest Beutler, MD

Department of Molecular and
Experimental Medicine
Scripps Research Institute
La Jolla, CA

Barry R. Bloom, PhD

Harvard School of Public Health
Boston, MA

Floyd E. Bloom, MD

Department of Neuropharmacology
Scripps Research Institute
La Jolla, CA

Noël Bouck, PhD

Department of Microbiology-Immunology
Northwestern University Medical School
Chicago, IL

Richard Bucala, MD, PhD

Picower Institute for Medical Research
Manhasset, NY

Mario R. Capecchi, PhD

Howard Hughes Medical Institute
University of Utah School of Medicine
Salt Lake City, UT

Anthony Cerami, PhD

Kenneth S. Warren Laboratories
Tarrytown, NY

Pierre Chambon, MD

Institut de Biologie Moléculaire et
Cellulaire
Strasbourg, France

Fred E. Cohen, MD, PhD

Department of Pharmacology
University of California, San Francisco
San Francisco, CA

R. John Collier, PhD

Department of Microbiology and
Molecular Genetics
Harvard Medical School
Boston, MA

Francis S. Collins, MD, PhD

National Human Genome Research
Institute
National Institutes of Health
Bethesda, MD

Max D. Cooper, MD

Howard Hughes Medical Institute
University of Alabama
Birmingham, AL

Ramzi Cotran, MD

Brigham and Women's Hospital
Harvard Medical School
Boston, MA

Shaun R. Coughlin, MD, PhD

Cardiovascular Research Institute
University of California, San Francisco
San Francisco, CA

Pedro M. Cuatrecasas, MD

Departments of Medicine and of
Pharmacology
University of California
San Diego, CA

Marilyn Gist Farquhar, PhD

Division of Cellular and Molecular
Medicine
University of California, San Diego
La Jolla, CA

Anthony S. Fauci, MD

National Institute of Allergy and
Infectious Diseases
Bethesda, MD

Douglas T. Fearon, MD

Wellcome Trust Immunology Unit
University of Cambridge School of Clinical
Medicine
Cambridge, United Kingdom

Judah Folkman, MD

Children's Hospital
Harvard Medical School
Boston, MA

David V. Goeddel, PhD

Chief Executive Officer
Tularik, Inc.
South San Francisco, CA

Paul Greengard, PhD

Laboratory of Molecular and Cellular
Neurosciences
Rockefeller University
New York, NY

Leonard Harrison, MD, DSC

The Walter and Eliza Hall Institute of
Medical Research
The Royal Melbourne Hospital
Victoria, Australia

David D. Ho, MD

Aaron Diamond AIDS Research Center
Rockefeller University
New York, NY

Leroy Hood, MD, PhD

Department of Molecular Biotechnology
University of Washington
Seattle, WA

Charles A. Janeway Jr., MD

Section of Immunobiology
Yale University School of Medicine
New Haven, CT

Tadamitsu Kishimoto, MD

Department of Medicine III
Osaka University Medical School
Osaka, Japan

Louis M. Kunkel, Francis A. Bourn

Children's Hospital
Harvard Medical School
Boston, MA

Philip Leder, MD

Department of Genetics
Harvard Medical School
Boston, MA

Jeffrey M. Leiden, MD, PhD

Department of Medicine and Pathology
The University of Chicago
Chicago, IL

Richard A. Lerner, MD

Scripps Research Institute
La Jolla, CA

Arnold J. Levine, PhD

Department of Molecular Biology
Princeton University
Princeton, NJ

Richard Locksley, MD, PhD

Department of Immunology
University of California
San Francisco, CA

Vincent T. Marchesi, MD, PhD

Boyer Center for Molecular Medicine
Yale University School of Medicine
New Haven, CT

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Molecular Medicine

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Commissioner for Patents
 P.O. Box 1450
 Alexandria, VA 22313-1450

DECLARATION BY INVENTORS UNDER 37 C.F.R. § 1.132

We, Beverly L. Davidson, Ph.D., Richard D. Anderson, Ronald E. Haskell, Ph.D., and Haibin Xia, Ph.D., hereby declare as follows:

(1) That we are co-inventors of the above-identified patent application, filed herewith, as well as U.S. Serial No. 09/521,524, which was filed March 8, 2000. The present application is a continuation of the 09/521,524 application.

(2) That Beverly Davidson, Ph.D., has been a faculty member of the University of Michigan (1990-1994) and The University of Iowa (1994-present) in Iowa City, Iowa. She currently holds the Roy J. Carver Professor Chair in Internal Medicine at The University of Iowa. In addition, she currently is the director of the Gene Transfer Vector Core at The University of Iowa. Her research involves, *inter alia*, the development of viral and non-viral vectors for gene transfer to the central nervous system. Dr. Davidson has published numerous articles in peer reviewed scientific journals in this area.

CERTIFICATE OF MAILING BY FIRST CLASS MAIL

I hereby certify under 37 CFR §1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

April 20, 2004

Date of Deposit

Theresa Popelar

Signature

Theresa Popelar

Typed or Printed Name of Person Signing Certificate

(3) That Richard D. Anderson is a co-founder and currently the President of ViraQuest, Inc., in North Liberty, Iowa. Mr. Anderson obtained a B.S. from Briar Cliff College, Sioux City, Iowa in 1985 and a B.S. (CLS) from the University of Iowa College of Medicine, Iowa City, Iowa in 1986. He worked as a medical researcher at the University of Iowa from 1987 through 2001. From 1994-2001, he also supervised the Gene Transfer Vector Core facility at the University of Iowa College of Medicine. Under Mr. Anderson's supervision, the Vector Core facility produced over 300 adenoviral constructs, the subject matter of which have been published in numerous peer-reviewed scientific journals. Mr. Anderson has collaborated with Dr. Ronald E. Haskell for eight years in the generation of recombinant adenovirus.

(4) That Ronald E. Haskell, Ph.D., is a co-founder and currently the Vice President of ViraQuest, Inc., in North Liberty, Iowa. Dr. Haskell obtained his Ph.D. from Colorado State University in Fort Collins, Colorado in 1995. Dr. Haskell was employed as a Postdoctoral Fellow from 1995-1998 and as a Research Investigator from 1998-2001 in the laboratory of Dr. Beverly Davidson at The University of Iowa. During this time, Dr. Haskell performed extensive experiments using adenoviral vectors. This work has been published in five articles in peer-reviewed scientific journals. As mentioned above, Dr. Haskell has collaborated with Richard D. Anderson for eight years in the generation of recombinant adenovirus.

(5) That Haibin Xia, Ph.D. was a Postdoctoral Fellow from 1997-2000 and a Research Investigator from 2000-2001 in Dr. Beverly Davidson's laboratory at The University of Iowa. Since 2002, Dr. Xia has been an Assistant Research Scientist in Dr. Davidson's laboratory. His research involves the development of viral vectors for gene transfer to the central nervous system. Dr. Xia has published articles in peer reviewed scientific journals in this area.

(6) That we have reviewed the Aoki *et al.* reference (*Mol. Medicine* 5:224-231, 1999) cited by the Examiner in Office Actions mailed during prosecution of U.S. Serial No. 09/521,524. We make the present Declaration in support of the patentability of the claims of the U.S. patent application filed herewith.

(7) That prior to June 15, 1999, we conceived of and prepared the backbone vectors and the shuttle plasmids recited in the claims of the present application, and worked diligently to use the backbone vectors and shuttle plasmids to produce recombinant adenovirus. The conception and production of the claimed shuttle and backbone plasmids, as well as the work to produce recombinant adenovirus, occurred in the United States.

(8) That **Exhibits A through H**, attached hereto and incorporated by reference herein, are factual evidence of conception and due diligence to reduction to practice of the invention in the United States prior to June 15, 1999.

(9) That **Exhibits A through H** disclose the preparation and testing of shuttle plasmids and backbone vectors such as those recited in the claims of the attached application. As is common in research laboratories, these shuttle and backbone constructs are designated by alternate identifiers. For example, the presently claimed shuttle plasmids and backbone vectors are identified in the attached exhibits as follows:

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Shuttle plasmids

<i>Designation</i>	<i>Large scale preparation</i>	<i>Alternate designation</i>
pAd5 RSV K-NpA	#779	Pac Ad5RSV K-NpA Ad5RSV K-NpA (PacI) pAd5RSVK-NpA(PacI) pacIAd5RSVK-NpA
pAd5 CMV K-NpA	#780	Pac Ad5CMV K-NpA Ad5cmvkNpA (PacI) pAd5CMVK-NpA(PacI) pacIAd5CMVK-NpA
Ad5 CMV hGFP		
pPACI CMV EGFP	#809	pac CMV EGFP pPACI CMV EGFP DH5alpha Ad5CMV EGFP PacI pPacICMVEGFPpA#3
Ad5 RSV hGFP		
pPACI RSV EGFP	#810	pac RSV EGFP pPACI RSV EGFP DH5alpha PacI RSV EGFP pPacIRSVEGFPpA#8 pacIAd5R\$V EGFP AD5R\$V EGFP

Intermediate plasmid

<i>Designation</i>	<i>Alternate designation</i>
del 0-1 #779 5	DELTA 0-1 779#5

Backbone plasmid

<i>Designation</i>	<i>Large scale preparation</i>	<i>Alternate designation</i>
del0-1sub360	#784, #849	DEL 0-1 SUB 360 #11 del0-1sub360 #3 del 0-1 sub 360 DH5alpha del sub 360 JM110 del sub 360 del0-1sub360#2 JM110

(10) That **Exhibit A** includes photocopies of three pages from the laboratory notebook of inventor Richard Anderson. While the dates on these pages have been blacked out, the original of each page is dated prior to the effective date of the Aoki *et al.* reference. These three pages refer to construction of the shuttle vectors pAd5 RSV K-NpA and pAd5 CMV K-NpA. Specifically, the first page of **Exhibit A** refers to engineering of a PacI site into the shuttle vectors, and also indicates that the vectors were prepared in large scale. The second page includes diagrams of these vectors, and also indicates the construction of the intermediate plasmid to be used in construction of the backbone. The intermediate plasmid is based on shuttle plasmid #779, but is devoid of map units 0-1 of the Ad genome. The third page indicates that the intermediate plasmid does lack map units 0-1. In addition, the third page includes a diagram of a method for making a backbone plasmid (indicated by asterisk) that lacks Adenovirus map units 0 to 9.2 and contains map units 9.2 to 100. *Thus, shuttle and backbone vectors were conceived of, and shuttle vectors were prepared, prior June 15, 1999.*

(11) That **Exhibit B** includes photocopies of two sequence chromatograms. While the dates on these chromatograms have been blacked out, the original of each page is dated prior to the effective date of the Aoki *et al.* reference. The first chromatogram shows the sequence of an intermediate plasmid that was prepared from a shuttle plasmid for use in making the backbone plasmid. The second chromatogram shows the sequence of a backbone plasmid. *Thus, shuttle, intermediate, and backbone vectors had been constructed, prepared in large scale, and sequenced prior to June 15, 1999.*

(12) That **Exhibit C** is a photocopy of a data sheet for a transfection carried out by inventor Richard Anderson, as indicated by the initials RDA entered in the space for "Initials of Transfector." While the dates on this page have been blacked out, the original page is dated prior to the effective date of the Aoki *et al.* reference. The transfection data sheet indicates that PacI-digested del 0-1sub360 #3 was used as a backbone plasmid, and the plasmids Ad5 RSV hGFP and Ad5 CMV hGFP were used as shuttles. In addition, the notes in the margin refer to the presence of green cells after the transfection, indicating that the inventors were successful in

using the cloning system to make recombinant virus. *Thus, the backbone and shuttle plasmids recited in the present claims were constructed and were used to make recombinant virus prior to June 15, 1999.*

(13) That **Exhibit D** is a photocopy of a page from the laboratory notebook of Richard Anderson. While the dates on this page have been blacked out, the original page is dated prior to the effective date of the Aoki *et al.* reference. This page refers to backbone plasmid del0-1sub360. In addition, this page indicates that inventor Ronald Haskell generated two shuttle vectors, designated pac CMV EGFP and pac RSV EGFP. Diagrams of these two vectors, which are shown to include map units 0-1 and 9.2-16 of the Ad genome, also are provided on this page. *Thus, the presently claimed shuttle and backbone plasmids were conceived of and prepared prior to June 15, 1999.*

(14) That **Exhibit E** is a printout of two pages from a spreadsheet containing information from large scale preparations of various plasmids. While the dates on these pages have been blacked out, the original pages are dated prior to the effective date of the Aoki *et al.* reference. The first page of this spreadsheet lists various shuttle plasmids, including Ad5RSV K-Npa, Ad5cmvkNpA, pPAC1 CMV EGFP, and pPAC1 RSV EGFP. In addition, this page lists the intermediate plasmid del 0-1 #779 5, and the backbone plasmid del 0-1 sub 360 DH5alpha. *Thus, a number of shuttle and backbone plasmids were prepared in large scale prior to June 15, 1999.*

(15) That **Exhibit F** is a photocopy of a seminar announcement distributed to University of Iowa faculty. The presentation was entitled ““Born to be Wild-type-Free”: New Methods for Adenovirus Generations [sic],” and was given by inventor Richard D. Anderson. While the date on this page has been blacked out, the original announcement was printed prior to the effective date of the Aoki *et al.* reference. In his presentation, inventor Anderson discussed the cloning system recited in the present claims. *Thus, the present invention was conceived of and well developed prior to June 15, 1999.*

(16) That **Exhibit G** includes photocopies of five pages from the laboratory notebook of Richard Anderson. While the dates on these pages have been blacked out, the original pages are dated prior to the effective date of the Aoki *et al.* reference. The pages in **Exhibit G** refer to a transfection using the del sub 360 backbone plasmid and the pPACI RSV EGFP DH5alpha and pPacI CMV EGFP DH5alpha shuttle plasmids. According to the first page, this transfection was carried out to make virus. The second page of this exhibit states that the transfection resulted in green cells, indicating that recombination had occurred. The third page refers to another experiment using the backbone plasmid del sub 360 and the shuttle plasmid PacI RSV EGFP. The fourth page indicates that green cells also were observed as a result of this transfection.

Thus, the cloning system as recited in the present claims was conceived of prior to publication of the Aoki et al. reference, and the inventors exhibited due diligence in reducing the invention to practice such that it was successfully used prior to June 15, 1999.

(17) That **Exhibit H** is a print out of a three-page document summarizing the cloning system of the present invention. The document was originally prepared by inventor Richard Anderson. While the dates on this document have been blacked out, the original document is dated prior to the effective date of the Aoki *et al.* reference. The first page of **Exhibit H** discloses the method used to generate the shuttle plasmids pAd5RSVK-NpA(PacI) and pAd5CMVK-NpA(PacI), as well as the del0-1sub360 backbone plasmid. The second page provides details of the method used to generate the shuttle plasmids pPacICMVEGFPpA#3 and pPacIIRSVEGFPpA#8. The second and third pages of this exhibit also disclose the method used to transfect HEK293 cells with the #810 shuttle plasmid and the del0-1sub360 backbone plasmid. *Thus, the shuttle vectors and backbone plasmids of the present invention had been prepared and used for transfection experiments prior to June 15, 1999.*

(18) We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so

Applicant : Beverly L. Davidson et al.
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made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

4/08/04
Date

Beverly L. Davidson
Beverly L. Davidson, Ph.D.

Date

Richard D. Anderson

Date

Ronald E. Haskell, Ph.D.

4-9-04
Date

Haibin Xia
Haibin Xia, Ph.D.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Commissioner for Patents
P.O. Box 1450
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Date of Deposit April 20, 2004

Signature Theresa Payne

Typed or Printed Name of Person Signing Certificate

(3) That Richard D. Anderson is a co-founder and currently the President of ViraQuest, Inc., in North Liberty, Iowa. Mr. Anderson obtained a B.S. from Briar Cliff College, Sioux City, Iowa in 1985 and a B.S. (CLS) from the University of Iowa College of Medicine, Iowa City, Iowa in 1986. He worked as a medical researcher at the University of Iowa from 1987 through 2001. From 1994-2001, he also supervised the Gene Transfer Vector Core facility at the University of Iowa College of Medicine. Under Mr. Anderson's supervision, the Vector Core facility produced over 300 adenoviral constructs, the subject matter of which have been published in numerous peer-reviewed scientific journals. Mr. Anderson has collaborated with Dr. Ronald E. Haskell for eight years in the generation of recombinant adenovirus.

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(7) That prior to June 15, 1999, we conceived of and prepared the backbone vectors and the shuttle plasmids recited in the claims of the present application, and worked diligently to use the backbone vectors and shuttle plasmids to produce recombinant adenovirus. The conception and production of the claimed shuttle and backbone plasmids, as well as the work to produce recombinant adenovirus, occurred in the United States.

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Shuttle plasmids

<i>Designation</i>	<i>Large scale preparation</i>	<i>Alternate designation</i>
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pAd5 CMV K-NpA	#780	Pac Ad5CMV K-NpA Ad5cmvkNpA (PacI) pAd5CMVK-NpA(PacI) pacIAd5CMVK-Npa
Ad5 CMV hGFP		
pPACI CMV EGFP	#809	pac CMV EGFP pPACI CMV EGFP DH5alpha Ad5CMV EGFP PacI pPacICMVEGFPpA#3
Ad5 RSV hGFP		
pPACI RSV EGFP	#810	pac RSV EGFP pPACI RSV EGFP DH5alpha PacI RSV EGFP pPacIRSVEGFPpA#8 pacIAd5RSV EGFP AD5RSV EGFP

Intermediate plasmid

<i>Designation</i>	<i>Alternate designation</i>
del 0-1 #779 5	DELTA 0-1 779#5

Backbone plasmid

<i>Designation</i>	<i>Large scale preparation</i>	<i>Alternate designation</i>
del0-1sub360	#784, #849	DEL 0-1 SUB 360 #11 del0-1sub360 #3 del 0-1 sub 360 DH5alpha del sub 360 JM110 del sub 360 del0-1sub360#2 JM110

(10) That **Exhibit A** includes photocopies of three pages from the laboratory notebook of inventor Richard Anderson. While the dates on these pages have been blacked out, the original of each page is dated prior to the effective date of the Aoki *et al.* reference. These three pages refer to construction of the shuttle vectors pAd5 RSV K-NpA and pAd5 CMV K-NpA. Specifically, the first page of **Exhibit A** refers to engineering of a PacI site into the shuttle vectors, and also indicates that the vectors were prepared in large scale. The second page includes diagrams of these vectors, and also indicates the construction of the intermediate plasmid to be used in construction of the backbone. The intermediate plasmid is based on shuttle plasmid #779, but is devoid of map units 0-1 of the Ad genome. The third page indicates that the intermediate plasmid does lack map units 0-1. In addition, the third page includes a diagram of a method for making a backbone plasmid (indicated by asterisk) that lacks Adenovirus map units 0 to 9.2 and contains map units 9.2 to 100. *Thus, shuttle and backbone vectors were conceived of, and shuttle vectors were prepared, prior June 15, 1999.*

(11) That **Exhibit B** includes photocopies of two sequence chromatograms. While the dates on these chromatograms have been blacked out, the original of each page is dated prior to the effective date of the Aoki *et al.* reference. The first chromatogram shows the sequence of an intermediate plasmid that was prepared from a shuttle plasmid for use in making the backbone plasmid. The second chromatogram shows the sequence of a backbone plasmid. *Thus, shuttle, intermediate, and backbone vectors had been constructed, prepared in large scale, and sequenced prior to June 15, 1999.*

(12) That **Exhibit C** is a photocopy of a data sheet for a transfection carried out by inventor Richard Anderson, as indicated by the initials RDA entered in the space for "Initials of Transfector." While the dates on this page have been blacked out, the original page is dated prior to the effective date of the Aoki *et al.* reference. The transfection data sheet indicates that PacI-digested del 0-1sub360 #3 was used as a backbone plasmid, and the plasmids Ad5 RSV hGFP and Ad5 CMV hGFP were used as shuttles. In addition, the notes in the margin refer to the presence of green cells after the transfection, indicating that the inventors were successful in

using the cloning system to make recombinant virus. *Thus, the backbone and shuttle plasmids recited in the present claims were constructed and were used to make recombinant virus prior to June 15, 1999.*

(13) That **Exhibit D** is a photocopy of a page from the laboratory notebook of Richard Anderson. While the dates on this page have been blacked out, the original page is dated prior to the effective date of the Aoki *et al.* reference. This page refers to backbone plasmid del0-1sub360. In addition, this page indicates that inventor Ronald Haskell generated two shuttle vectors, designated pac CMV EGFP and pac RSV EGFP. Diagrams of these two vectors, which are shown to include map units 0-1 and 9.2-16 of the Ad genome, also are provided on this page. *Thus, the presently claimed shuttle and backbone plasmids were conceived of and prepared prior to June 15, 1999.*

(14) That **Exhibit E** is a printout of two pages from a spreadsheet containing information from large scale preparations of various plasmids. While the dates on these pages have been blacked out, the original pages are dated prior to the effective date of the Aoki *et al.* reference. The first page of this spreadsheet lists various shuttle plasmids, including Ad5RSV K-Npa, Ad5cmvkNpA, pPAC1 CMV EGFP, and pPAC1 RSV EGFP. In addition, this page lists the intermediate plasmid del 0-1 #779 5, and the backbone plasmid del 0-1 sub 360 DH5alpha. *Thus, a number of shuttle and backbone plasmids were prepared in large scale prior to June 15, 1999.*

(15) That **Exhibit F** is a photocopy of a seminar announcement distributed to University of Iowa faculty. The presentation was entitled "Born to be Wild-type-Free": New Methods for Adenovirus Generations [sic]," and was given by inventor Richard D. Anderson. While the date on this page has been blacked out, the original announcement was printed prior to the effective date of the Aoki *et al.* reference. In his presentation, inventor Anderson discussed the cloning system recited in the present claims. *Thus, the present invention was conceived of and well developed prior to June 15, 1999.*

(16) That **Exhibit G** includes photocopies of five pages from the laboratory notebook of Richard Anderson. While the dates on these pages have been blacked out, the original pages are dated prior to the effective date of the Aoki *et al.* reference. The pages in **Exhibit G** refer to a transfection using the del sub 360 backbone plasmid and the pPACI RSV EGFP DH5alpha and pPacI CMV EGFP DH5alpha shuttle plasmids. According to the first page, this transfection was carried out to make virus. The second page of this exhibit states that the transfection resulted in green cells, indicating that recombination had occurred. The third page refers to another experiment using the backbone plasmid del sub 360 and the shuttle plasmid PacI RSV EGFP. The fourth page indicates that green cells also were observed as a result of this transfection. *Thus, the cloning system as recited in the present claims was conceived of prior to publication of the Aoki et al. reference, and the inventors exhibited due diligence in reducing the invention to practice such that it was successfully used prior to June 15, 1999.*

(17) That **Exhibit H** is a print out of a three-page document summarizing the cloning system of the present invention. The document was originally prepared by inventor Richard Anderson. While the dates on this document have been blacked out, the original document is dated prior to the effective date of the Aoki *et al.* reference. The first page of **Exhibit H** discloses the method used to generate the shuttle plasmids pAd5RSVK-NpA(PacI) and pAd5CMVK-NpA(PacI), as well as the del0-1 sub360 backbone plasmid. The second page provides details of the method used to generate the shuttle plasmids pPacICMVEGFPpA#3 and pPacIRSVEGFPpA#8. The second and third pages of this exhibit also disclose the method used to transfect HEK293 cells with the #810 shuttle plasmid and the del0-1 sub360 backbone plasmid. *Thus, the shuttle vectors and backbone plasmids of the present invention had been prepared and used for transfection experiments prior to June 15, 1999.*

(18) We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so

Applicant : Beverly L. Davidson et al.
Serial No. :
Filed : Herewith
Page : 8 of 8

Attorney's Docket No.: 17023-005001 / 00015

made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date

4/6/04

Date

4/7/04

Date

Date

60207120.doc

Beverly L. Davidson, Ph.D.

Richard D. Anderson

Richard D. Anderson

Ronald E. Haskell

Ronald E. Haskell, Ph.D.

Haibin Xia, Ph.D.

Construction of PacI site into shuttlevector.

pAd5 KSV K-NpA

2 oligo Ad PacI Rev

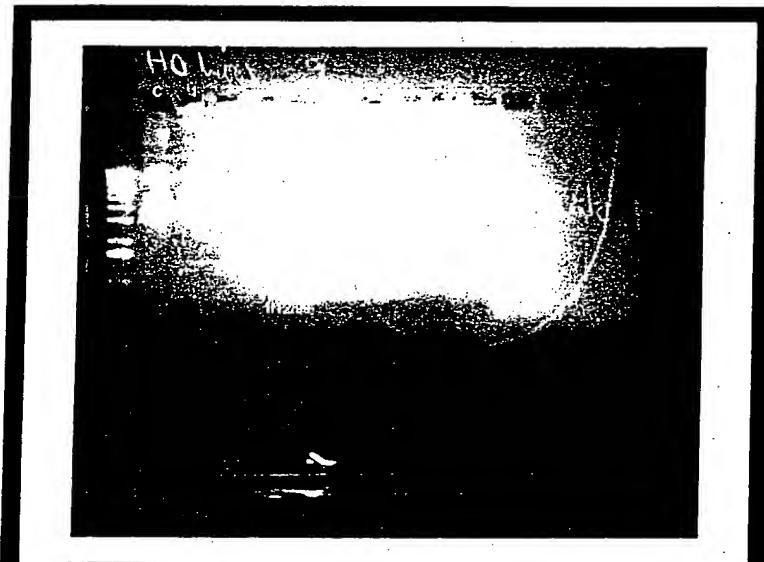
Ad PacI rev

pAd5cmV K-NpA

Shuttle quickchange kit by R. Haskell

manipulated by P. Staber

PacI digest



large scale
prep of #9
#15 #7

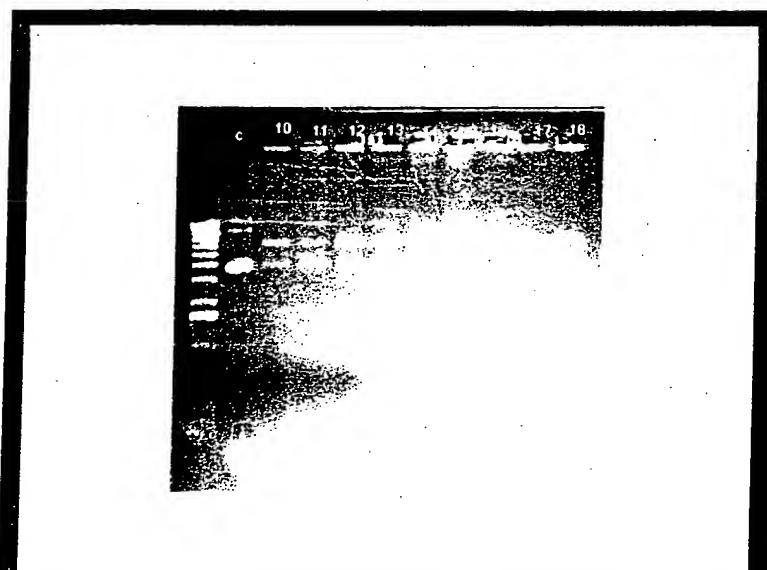
PacI
HO-1 KSV
NEB

PacI
HO-1 cmV
NEB

* To be used for
"gentless"
"α" + ori

for making

* O-1 minus
plasmid for
recombinations.

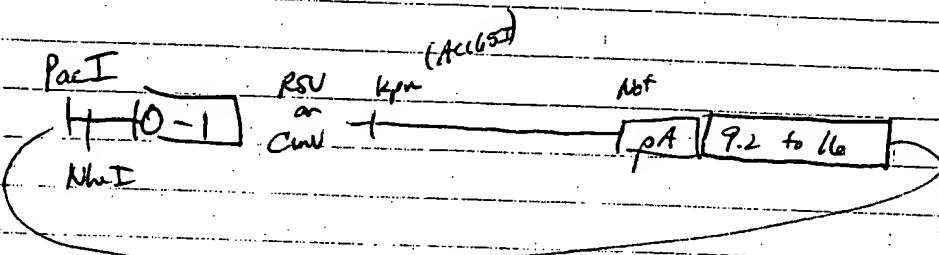


#779 Pac Ad5RSV K-Npt 0.3ug/ml

part digest
3ul DNase ✓

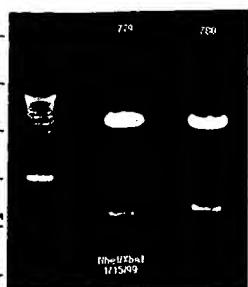
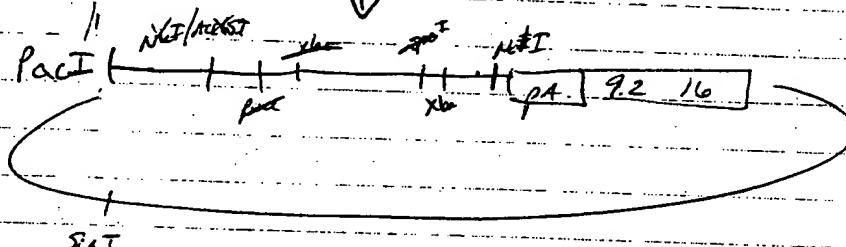
#780 Pac Ad5CmV K-Npt 0.4ug/ml

3ul O1 ✓
2ul PacI
22ul H₂O ✓



digest both in ~~PstI~~ / ~~XbaI~~ / ~~NheI~~ XbaI
Fill in ~~T4~~ DNA poly + ligate

NheI/XbaI
will ligate
together to
make BglI site



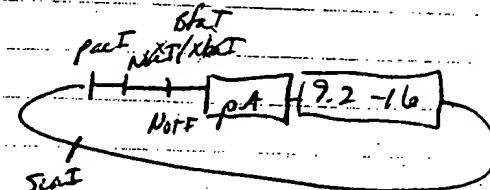
10ul #779 ✓
3ul MO
1.5ul NheI
1ul XbaI
14.5ul H₂O ✓

10ul #780 ✓ 1hr 57°C
3ul MO
1.5ul NheI
1ul XbaI
14.5ul H₂O ✓

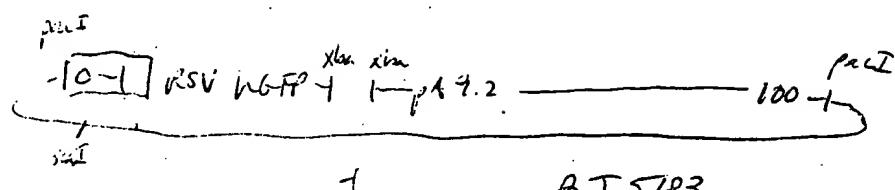
Isolate frangal EtOH ppt and ligate
resuspend in 22ul H₂O
use 5ul for ligation

8.40ml 5ul DNA
1.2ul 10X ligO
1.5ul ligation
1/10.5ul H₂O

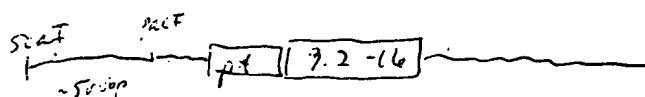
RT ~1 hour



→ *hor nobis
accedit*



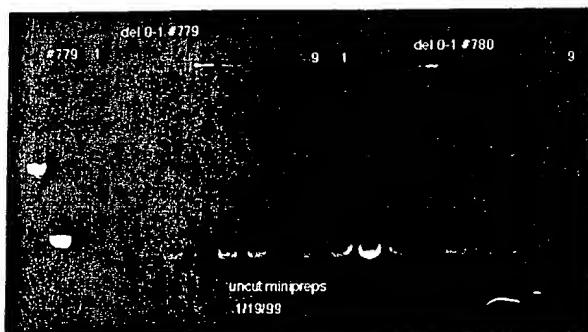
BJ583



1



11



100ul 0452
+
15ul IgG

4 tes. 1. #779
2. #780
3. HSV only

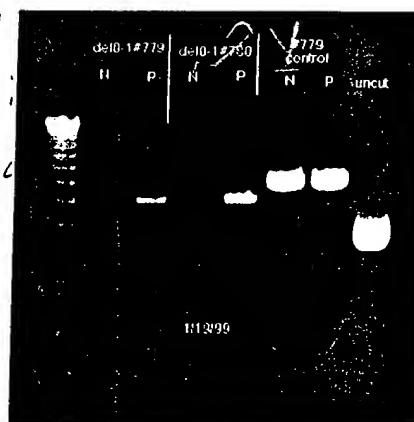
-Mungroon A.O.-1 779
A.O.-1 750

all have 1-9
deletion 1-5

AO-1 723 #5 Sub
AO-1 720 #9

778 a2 central

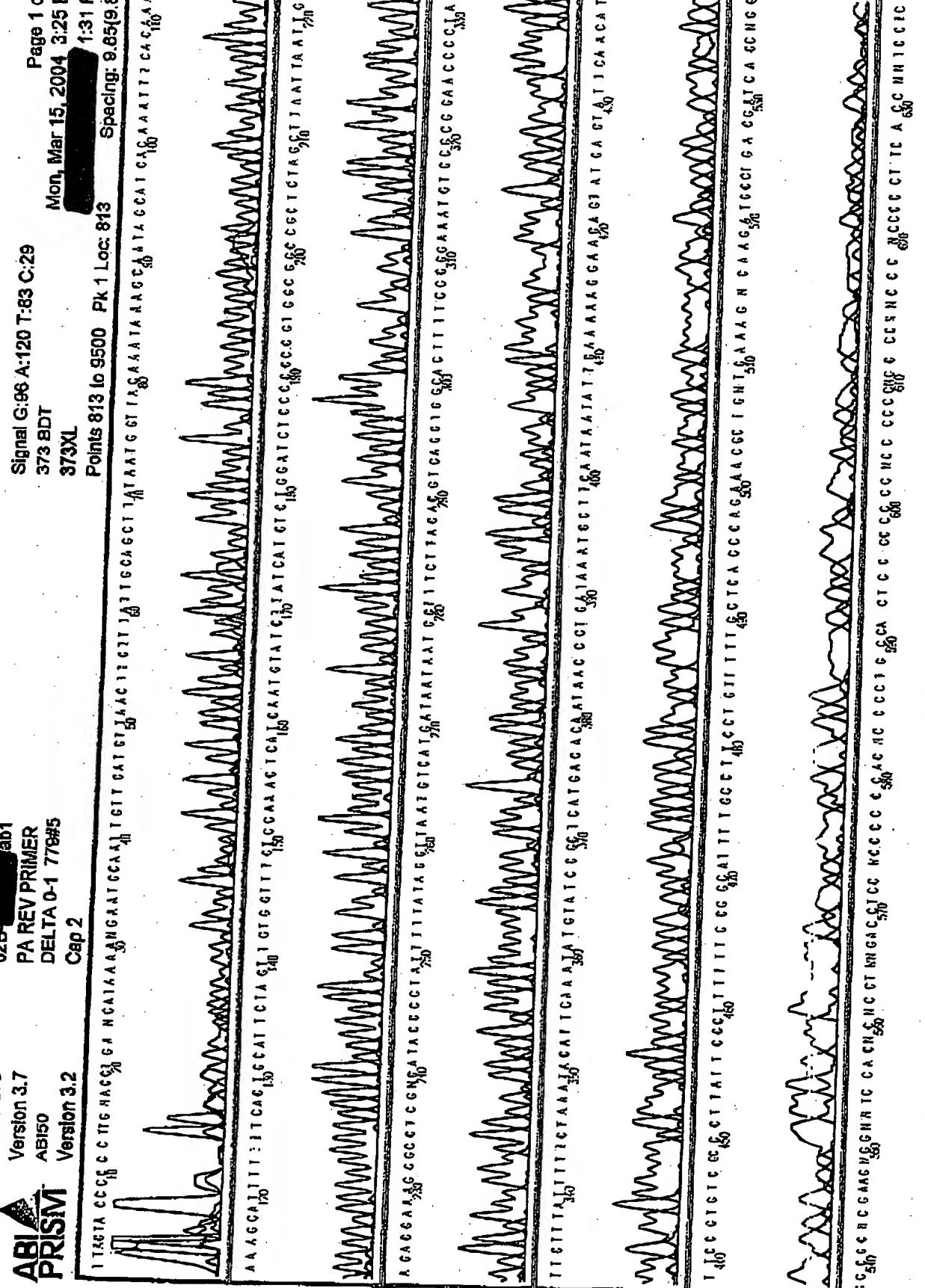
Sig OK
P. Arw.



✓ Sul DNA
3rd 10x0
✓ Sul Enzyme
2nd 4²⁰

ABI
PRISM
 Model 373
 Version 3.7
 ABI50
 Version 3.2
 02B-
 ab1
 REV PRIMER
 DELTA 0-1 77845
 Cap 2

Signal G:96 A:120 T:83 C:29
 373 BDT
 373XL
 Points 813 to 9500 Pk 1 Loc: 813
 Mon, Mar 15, 2004 3:25 PM
 [REDACTED] 1:31 PM
 Spacing: 0.85(9.85)
 1 TACTA CCCCTTGGACCGA
 2 MCATAAAGGAACTGCA
 3 TGTCTTACCTTACCTTAC
 4 TGGTTGCTTCTTCTTCTT
 5 CAAACCTTCTTCTTCTTCT
 6 GAGGAACTTCTTCTTCTT
 7 TCTTCTTCTTCTTCTTCTT
 8 TCTTCTTCTTCTTCTTCTT
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 98 TCTTCTTCTTCTTCTTCTT
 99 TCTTCTTCTTCTTCTTCTT
 100 TCTTCTTCTTCTTCTTCTT



Transfection for Recombinant Virus

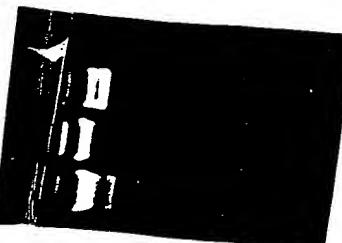
Initials of Transfector: RD

Transfection Start Date: _____ Date fed: _____ Date neutral red: _____ Date picked: _____

Comments:

part digested minicup DNA del 0.1 sub 300 #3
protoplasts E7H off suspended in 20ml
sterile H2O used 2 previous shuttle kb/T
linear plasmids.

三



Soil digest	175ml	462g	(50ml)
100ml H ₂ O	1.5ml	35.4ml	
5.2nd 50 ml	3.5ml	all 0.1 ml to 35ml	35ml
340ml 100% EtOH			
10ml 80% EtOH	5.0ml	1.5ml	
3rd 2nd 70% EtOH wash	25ml	8.0ml	25ml

Shri Ganesh
Ganesha
remained and
spoke to him.

line 1st.

Removed 100ml of media colored
to fresh plate of 223 cells
at 2:20 p.m.

Restriction Digest to test plaque forming ability of "R+R" vector Transfection of old shuttles.

PacI 1. #784 del 0-1 sub 360 0.2ug/l [REDACTED] ^{Expt 10 ug 10%}
3rd cell 4th

NheI 2. #685 pfd RSV hEGFP 0.3ug/l [REDACTED] 3rd 33rd

NheI 3. #751 pfd RSV K lacZ 0.4ug/l [REDACTED] 3rd ✓ 17th ✓
(ut lacZ by R+)

[REDACTED] There need to be transfected.

Note: Ron Haskell made 2 new clones

pac CMV EGFP
pac RSV EGFP

There will be used to make clones not the above prototypes

pac NheI
+ + 0-1 [RSV] EGFP pA 9.2-16
" " [CMV] " " "

There will be digested to

pacI or NheI to determine if other sit. cause problem in recombination and virus production

Large gene prep

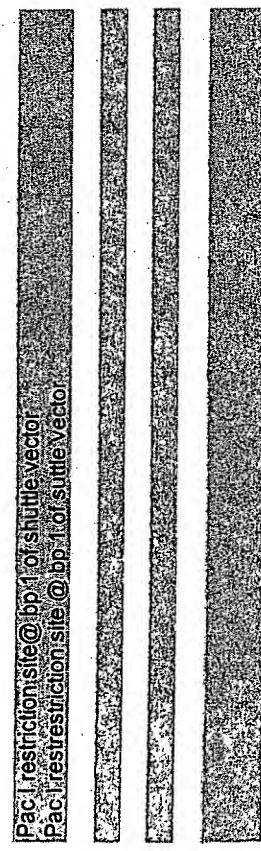
185 PTBE#6	2.700	R. Anderson	33883
186 PTET-On#7	7.300	R. Anderson	33883
190 psub360d ³ #6	9.100	R. Anderson	33890
191 psub360d ³ #18	8.000	R. Anderson	33890
215 pAd5CMV h GFP#5	1.150 2.130	RDH	33946
219 SK fiblink #6	0.570 2.036	KZ/RDA	33950
233 pIND	6.130 2.059	Ron	1/15/1997
234 pIND lacZ	6.110 2.032	Ron	1/15/1997
235 pVg RXR	5.020 2.068	Ron	1/15/1997
295 AdRSV Kpn-Not	0.490 2.45	PDS/RDA	34051
383 Ad mcs PA #9 in JM110	0.470 2.385	PDS	34158
384 Ad RSV K-N #5 in JM110	0.660 2.237	PDS	34158
385 Ad CMV K-N #13 in JM110	0.575 2.300	PDS	34158
491 pTG SN53	1.580	1.860 Haibin	34366
533 pTRE Ela	0.180	1.712 RDA	34429
548 Ad5.5 ires hGFP#5	0.860	1.838 RDA	34444
560 Ad5 cmVires hGFP #11	0.670	1.898 RDA	34457
598 pTGSN53E4delta#2	1.210	1.861 RDA	34493
609 pTGBH1/E4delta #13	0.170	1.894 RDA	34506
616 pTGSN53/swal	1.400	1.830 Haibin	34515
621 pTGBH1/Swal-1	0.450	1.799 Haibin	34522
622 pTGBH1/Swal-2	0.450	1.817 Haibin	34522
638 pTG3602/Swal	0.270	1.765 Haibin	34541
651 Ad5RSV CEB	0.670	1.727 RDA	34563
661 pTG360	0.290	1.611 Haibin	34577
779 Ad5RSV K-NPA (Pac) #9	0.330	1.941 RDA	34710
780 Ad5RSV K-NPA (Pac) #15	0.370 >2.00	RDA	34710
784 pGK-GFP	0.880	1.832 RDA	34726
796 del/0-#7795	0.450	1.917 RDA	34743
809 pPACT-GVNECFP	0.470	1.779 Ron	34756
810 pPACT-RSVEGFP	0.540	1.836 Ron	34758

87xxbp, pTGT1696 not2, Spe2 cut then ligate

Ad5 wt with Kpn1 religated p35 lacZ to use for new ires hGFP vector

5mal deleted E4 E.coli recombined with pTGBH1

Pac1 restriction site@bp1 of shuttle vector
Pac1 restriction site@bp1 of shuttle vector



"Born to be Wild-type Free" New Methods for Adenovirus Generations

**Gene Transfer Research
Group Seminar**

Presenter:

Richard D. Anderson

12:00-1:00 PM

**1-561 BSB
(MacEwen Conference Room)**

Lunch will be provided



Transfection to make virus from 2 plasmids

backbone #849 del/sub 360 JM110 0.2 ug/lul

shuttle #810 pPACI RSU EGFP DH5 α 0.5 ug/lul

Shuttle #809 pPACI CMV EGFP DH5 α 0.5 ug/lul

10ug of shuttle plus 1ug backbone

digested in fact for 1 hour

Then Ca^{++} ppt into 293 cells let go for

3 days then harvest.

✓ 10ul Shuttle
3ul 10x C

✓ 1ul PacI
✓ 5ul H_2O

✓ 10ul Backbone
3ul 10x C

2ul PacI
✓ 15ul H_2O

37°C 1 hour.

Ron took pictures 810, 809 + 849 showed an increase in the # of green cells after 72 hrs.

There appeared to be cpe. Talked to Habsin and he thought and plates were harvested at 3 days maybe to early based on 72 hrs frozen thawed x3 then E-roli virus production and placed on A549 cells. No green after 24 hours.

Repeat and wait for complete cpe ~5-7 days.

plate used > 80% confluent
A Ca²⁺P left on for only ~2 hours
before changing media.

picture in scope pdf folder under
R&R heading.

[REDACTED] #809 + RTR lysate on 213 cells shows 3
green cells Today.

First evidence of resemination by this
method.

Ran out lysate
on Tues a.m. Took pictures there were a total of 6 green
cells will add fresh media to plates
and look over the weekend.

[REDACTED] Added 2mls fresh 10% FBS/pen/st.

R&R measurement w/ 293 cells

#849 del sub 360 0.2ug/ml Total = 4ug

#810 pact KSV 0.54ug/ml 3/2/89 ✓ 18.5ul - 10u

Bal 10x0

2ul pact

5ul H₂O

37°C 1 hour.

Heat kill 65°C 20min use w/ transfection

60mm plate 293

1 #849 only

2 849 plus 810

Ad CMV TLR 2 #3 (Zabor) cut = NheI

Transfected 31b360 viral DNA

RSVE048

810

pretty good transfection c #809 / R&R

"cells look healthy will take picture

and follow over the weekend to watch
for increase in "green".

picture to R&R folder [REDACTED] labeled (a) n24hrs

[REDACTED] picture on Davidson Server for 98 hrs.

72 hrs

[REDACTED] increase in "green" cell number and
different intensities of "green"

7 days

5 days

6 days

7 days

of cells appears to increase til day 4

at day 5 not much difference. day 6 great looking com-

very infected green cells without a center (plaque) with
a trailing of lighter green cells behind it.

[REDACTED] removed 200ul of media from #7 plate
on [REDACTED] added it to 100mm plate 283 cells
~ 60% confluent.

a.m. looked at plate briefly and saw green cells!!
Should be due to debris infiltration much from
the R&R spot on plate #7

2:00pm 4-6 "comets" on the 100mm plate
very clearly visibly infected 283 cells that
are expressing GFP.

Harvest plate #7 on [REDACTED] to make lysate for
particle amplification, let 100mm so until CSE

Restriction Digest to produce Recombinant Ad virus
using R&R system

already cut 1. Ad5 CMV flt4-eGFP NheI cut \sim 1ug/ml Persin
1/1000 8ul 2. Ad5 CMV MSV A #6 NheI cut \sim 1ug/ml Hoshi
1/1000 19ul 3. Ad5 CMV SC/CMV3/eGFP #83 NheI cut \sim 1ug/ml Camb.
4. Ad5 CMV eGFP pacI

1/1000

2ul NheI

RT/20°C

(X2) #849 del 5ab340 0.2ug/ml, 1/20ul

1/3ul 10x

2ul pacI

1/5ul H₂O

#2, 3 outside DNA sample - 20

PM 221

500ul H₂O

Shuttle plasmid

R&R backbone

vortex

25ul CacL₂

vortex

RT 25min

Add to 2mls 2% FBS/DMEM 2-4 hours

Change media to 4mls 10% FBS/DMEM

let go ~ 7 days

Watch GFP virus for increase in green after
3 days.

R\$R Adenovirus Recombination System

Shuttle vector construction:

pAd5CMVK-NpA#390 and pAd5RSVK-NpA#600 were used for the starting plasmids for the system. The EcoRI site of these shuttles was converted to a PacI site by the Quik changeTM site directed mutagenesis kit from Stratgene.

The primers used or this are as follows:

AdPacIfor 5'-AGGCCCTTCGTCTCAATTAATTAAGCTAGCATCATCAATA-3'

AdPacIrev 5'-TATTGATGATTGCTAGCTTAATTGAAGACGAAAGGCC-3'

Bold letters are the PacI restriction site underlines are the NheI site.

Quikchange carried out by Dr. Ron Haskell plasmids grown and CsCl purified:

#779 pAd5RSVK-NpA(PacI) [REDACTED]
 #780 pAd5CMVK-NpA(PacI) [REDACTED]

Production of deleted 0-1 map unit shuttles:

#779 and #780 digested with NheI and XbaI and re ligated on itself. This removes the 0-1 m.u. of the Ad5 genome that contains the left hand ITR and packaging signal. It also removes the promoter region RSVor CMV.

Only continued with one of these new plasmids from the #779 re ligation. The new shuttle is called del0-1#779#5 changed to del0-19.2-16. This plasmid was sequenced with the pAreV primer(5'-TTAAAAAACCTCCCCACCTCCCC-3') 02B-[REDACTED]

To make the R\$R backbone(del0-1sub360) the following plasmids were used:

pTGRSVhGFP This plasmid was produced by Lane Law by the E.coli recombination system from Transgene. pTG3601 was digested with Bgl II and co transformed into BJ5183 E.coli with pure viral DNA from Ad5RSVhGFP adenovirus particles. Colonies were screened by EcoRI restriction digest and the correct plasmid purified on CsCl gradient.

The del0-1backbone was created by using the E.coli system to delete the 0-1m.u.RSV promoter and the hGFP gene as follows. The pTGRSVhGFP was digested with XbaI and the del0-1 9.2-16 shuttle was digested with ScaI both of these plasmids were transformed into BJ5183 E.coli and minipreps isolated. del0-1sub360#11 from the BJ5183 E.coli was sequenced with the pAreV primer 20B-[REDACTED]. This DNA was then transformed into DH5 α E.coli and CsCl purified#784(methylated). Upon checking this plasmid by restriction digest with EcoRI it appeared there was a mutation at this site that should have been at position 30009bp of the Ad genome. Plasmid #784 was sequenced with E3for2 primer(5'-

GTCCAACCTACAGCGACCCACCCTAACAGAG-3') 11W [REDACTED] the sequence aligned with Ad5 dl309 sequence which is correct.

#784 was re transformed into JM110 E.coli [REDACTED] failed to give colonies at 37°C for 2 minutes during heat shock step of transformation. #784 plasmid was transfected into HEK293 cells cells were harvested 24 hours later and run on a western blot [REDACTED]. Lysates tested versus 4D2.5 fiber monoclonal and Sheep90 adenovirus polyclonal. Lysates from #784 gave positive band for fiber and had a similiar pattern for the sheep polyclonal as compared to purified virus particles. E.coli transformation into JM110 was repeated using 2 minutes at 42°C, as per suggestion of Dr. Hiabin Xia, during the heat shock step. Isolated 2 colonies and grew a large scale prep of del0-1sub360#2 JM110 changed name to del0-1sub360[R\$R backbone #849 [REDACTED]].

First Adenovirus recombination in HEK293 cells was carried out to make Ad5R\$V EGFP. The shuttle used for this virus was constructed as follows:

pacIAd5RSVK-NpA#779 and pacIAd5CMVK-NpA#780 were digested with XhoI/NotI and ligated to the XhoI/NotI fragment from Clonetech pEGFP-N1 accession# U55762 (cat.#6085-1). Minipreps were checked with SalI restriction digest. Large scale CsCl DNA was made from the positive clones pPacICMVEGFPpA#3 (#809)and pPacIRSVEGFPpA#8 (#810).

10 μ g of pacIAd5R\$V EGFP#810 [REDACTED] was digested in a 30 μ L reaction using 8 units of PacI restriction enzyme at 37°C for 1 hour. The PacI was then heat killed at 65°C for 20 minutes. Two micrograms of R\$R backbone #849 was digested as above [REDACTED].

HEK293 cells were plated at ~1.5x10⁶ cells per 60mm plate 24 hours pre-transfection in 10% FBS/DMEM P/S and incubated in 95% humidity 5% CO₂. The transfection protocol was carried out as per the traditional Ca⁺⁺ Phosphate method used in the Gene Transfer Vector Core. The R\$R backbone and the Ad5R\$V EGFP shuttle were added to 500 μ L of HEBS buffer pH 7.1 and briefly vortexed. 25 μ L of 2.5 M CaCl₂ was added to the tube and precipitant allowed to form at room temperature for 25 minutes. During this time period the media was changed on the transfection plate to 2% FBS/DMEM and placed in the incubator to equilibrate. The total precipitant was added drop wise to the 2 mLs of media. The transfection media was left on the cells for ~2.5-3 hours before changing to 10% FBS/DMEM and allowed to incubate for ~ 7 days. The number of green cells was monitored by fluorescence every 24 hours. At day 6 [REDACTED] 200 μ L of media was removed and added to a 50% confluent plate of HEK293 cells in a 100mm plate. Green cells were observed after 24 hours and cpe seen after 48 hours post infection with the 200 μ L of media.

10 150mm plates of HEK293 cells were plated at 2 x10⁶ cells per plate 3 days before infection with the media and cell lysate of the Ad5R\$V EGFP transfection plate. The infection was allowed to go for ~ 30 hours before harvesting. The infected cells were spun in a 50 mL Falcon tube and the media aspirated. The cell pellet was washed one time with 1x PBS and resuspended in 0.5 mL of 10 mM Tris pH 8.0 per plate collect. The cell suspension was freeze thawed 3 times in an ethanol dry ice bath and the cell lysate collected. The lysate was then passed over a CsCl gradient and the virus particles isolated. The new Ad5R\$V EGFP particles [REDACTED] were resuspended in an equal volume of 50% glycerol/1% BSA and stored at -20°C.

Viral DNA was isolated from 100 μ L of the Ad5R\$V EGFP particles [REDACTED] and PCR was carried out to determine the presence of "wild-type" E1 positive signal. The viral particles were incubated with an equal volume of 2x Pronase solution at 37°C for at least one hour. The sample was then phenol/Chloroformed extracted, NaCl2/EtOH precipitated and resuspended in 100 μ L H₂O. 5 μ L(~3.5 x10¹⁰ genomes) of this was used in a 50 μ L PCR reaction using E1for2/E1rev1 and E3for2/E3rev1 primers.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Beverly L. Davidson et al. Art Unit : 1648
Serial No. : 09/521,524 Examiner : Shanon A. Foley
Filed : March 8, 2000
Title : RAPID GENERATION OF RECOMBINANT ADENOVIRAL VECTORS

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P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF ELIZABETH N. KAYTOR, PH.D. UNDER 37 C.F.R. § 1.132

I, Elizabeth N. Kaytor, hereby declare as follows:

(1) That I am employed as a Technology Specialist in the Minneapolis office of Fish & Richardson P.C., P.A.

(2) That I obtained the April 1999 issue of *Molecular Medicine* from the Bio-Medical Library at the University of Minnesota in Minneapolis. A copy of the cover page of the April 1999 issue is attached. The cover page is date stamped June 15, 1999.

(3) I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

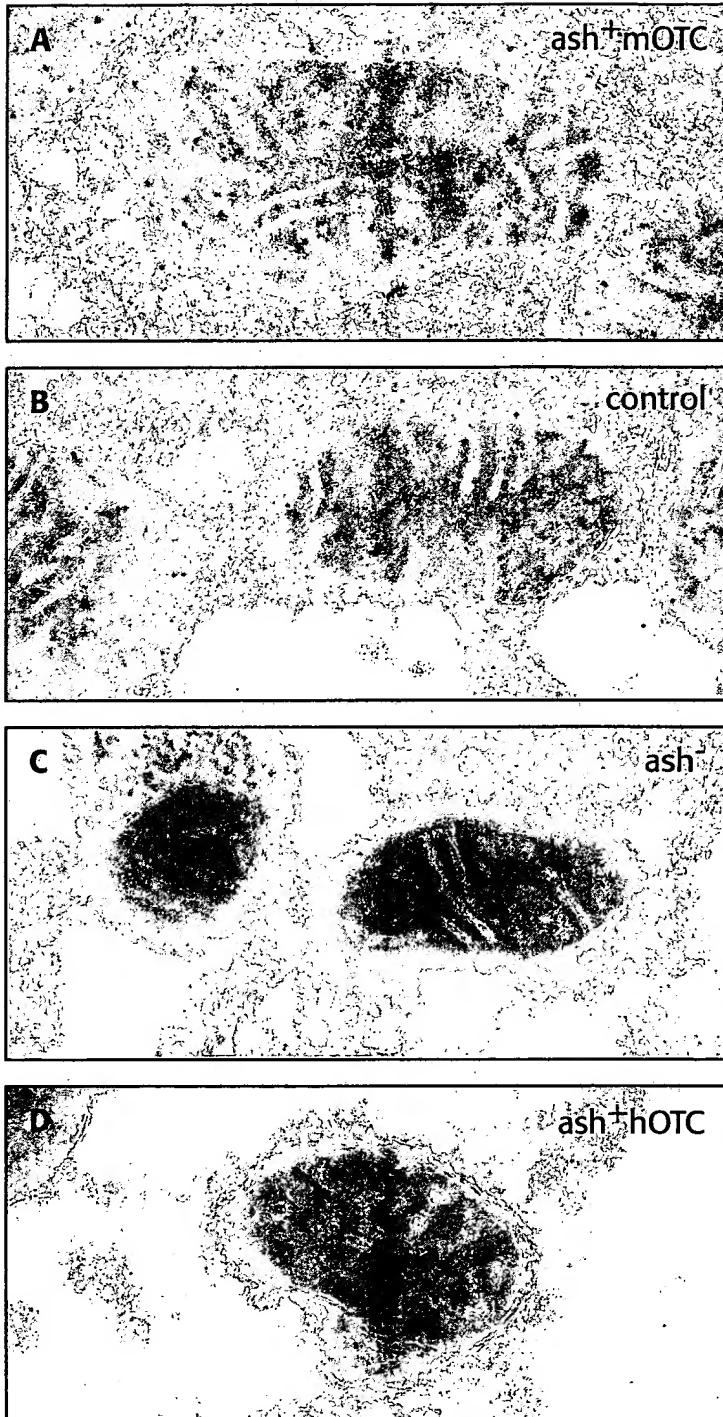
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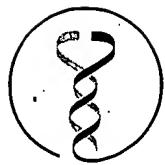
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EDITORS

EDITOR-IN-CHIEF

David Weatherall, FRS

*Institute of Molecular Medicine
John Radcliffe Hospital
Oxford, United Kingdom*

MANAGING EDITOR

Yvonne Cole, PhD

*Picower Institute for Medical Research
350 Community Drive
Manhasset, NY 11030*

CONTRIBUTING EDITORS

Frederick W. Alt, PhD

*Children's Hospital
Harvard Medical School
Boston, MA*

K. Frank Austen, MD

*Brigham and Women's Hospital
Harvard Medical School
Boston, MA*

Ernest Beutler, MD

*Department of Molecular and
Experimental Medicine
Scripps Research Institute
La Jolla, CA*

Barry R. Bloom, PhD

*Harvard School of Public Health
Boston, MA*

Floyd E. Bloom, MD

*Department of Neuropharmacology
Scripps Research Institute
La Jolla, CA*

Noël Bouck, PhD

*Department of Microbiology-Immunology
Northwestern University Medical School
Chicago, IL*

Richard Bucala, MD, PhD

*Picower Institute for Medical Research
Manhasset, NY*

Mario R. Capecchi, PhD

*Howard Hughes Medical Institute
University of Utah School of Medicine
Salt Lake City, UT*

Anthony Cerami, PhD

*Kenneth S. Warren Laboratories
Tarrytown, NY*

Pierre Chambon, MD

*Institut de Biologie Moléculaire et
Cellulaire
Strasbourg, France*

Fred E. Cohen, MD, PhD

*Department of Pharmacology
University of California, San Francisco
San Francisco, CA*

R. John Collier, PhD

*Department of Microbiology and
Molecular Genetics
Harvard Medical School
Boston, MA*

Francis S. Collins, MD, PhD

*National Human Genome Research
Institute
National Institutes of Health
Bethesda, MD*

Max D. Cooper, MD

*Howard Hughes Medical Institute
University of Alabama
Birmingham, AL*

Ramzi Cotran, MD

*Brigham and Women's Hospital
Harvard Medical School
Boston, MA*

Shaun R. Coughlin, MD, PhD

*Cardiovascular Research Institute
University of California, San Francisco
San Francisco, CA*

Pedro M. Cuatrecasas, MD

*Departments of Medicine and of
Pharmacology
University of California
San Diego, CA*

Marilyn Gist Farquhar, PhD

*Division of Cellular and Molecular
Medicine
University of California, San Diego
La Jolla, CA*

Anthony S. Fauci, MD

*National Institute of Allergy and
Infectious Diseases
Bethesda, MD*

Douglas T. Fearon, MD

*Wellcome Trust Immunology Unit
University of Cambridge School of Clinical
Medicine
Cambridge, United Kingdom*

Judah Folkman, MD

*Children's Hospital
Harvard Medical School
Boston, MA*

David V. Goeddel, PhD

*Chief Executive Officer
Tularik, Inc.
South San Francisco, CA*

Paul Greengard, PhD

*Laboratory of Molecular and Cellular
Neurosciences
Rockefeller University
New York, NY*

Leonard Harrison, MD, DSc

*The Walter and Eliza Hall Institute of
Medical Research
The Royal Melbourne Hospital
Victoria, Australia*

David D. Ho, MD

*Aaron Diamond AIDS Research Center
Rockefeller University
New York, NY*

Leroy Hood, MD, PhD

*Department of Molecular Biotechnology
University of Washington
Seattle, WA*

Charles A. Janeway Jr., MD

*Section of Immunobiology
Yale University School of Medicine
New Haven, CT*

Tadamitsu Kishimoto, MD

*Department of Medicine III
Osaka University Medical School
Osaka, Japan*

Louis M. Kunkel, PhD

*Children's Hospital
Harvard Medical School
Boston, MA*

Philip Leder, MD

*Department of Genetics
Harvard Medical School
Boston, MA*

Jeffrey M. Leiden, MD, PhD

*Department of Medicine and Pathology
The University of Chicago
Chicago, IL*

Richard A. Lerner, MD

*Scripps Research Institute
La Jolla, CA*

Arnold J. Levine, PhD

*Department of Molecular Biology
Princeton University
Princeton, NJ*

Richard Locksley, MD, PhD

*Department of Immunology
University of California
San Francisco, CA*

Vincent T. Marchesi, MD, PhD

*Boyer Center for Molecular Medicine
Yale University School of Medicine
New Haven, CT*

Philippa Marrack, PhD
National Jewish Center for Immunology
and Respiratory Medicine
Denver, CO

Adolfo Martinez-Palomo, MD
Centro de Investigacio y de Estudios
Avanzados
Instituto Politecnico Nacional Mexico

Hugh O. McDevitt, MD
Microbiology and Immunology
Stanford University School of Medicine
Stanford, CA

Louis H. Miller, MD
Laboratory of Malaria Research
National Institutes of Health
Bethesda, MD

N. Avrion Mitchison, PhD
Forschungslaboratorium
Deutsches RheumaForschungZentrum
Berlin, Germany

Salvador Moncada, MD, FRS
Wellcome Research Laboratories
Wellcome Foundation UK
Beckenham, United Kingdom

Elizabeth G. Nabel, MD
Division of Cardiology
The University of Michigan Medical Center
Ann Arbor, MI

Carl F. Nathan, MD
Microbiology, Immunology and Medicine
Weill Medical College of Cornell
University
New York, NY

David G. Nathan, MD
Dana-Farber Cancer Institute
Boston, MA

Bert W. O'Malley, MD
Département of Cell Biology
Baylor College of Medicine
Houston, TX

Stuart H. Orkin, MD
Children's Hospital
Harvard Medical School
Boston, MA

Athanassios G. Papavassiliou, MD, PhD
Department of Biochemistry
University of Patras School of Medicine
Patras, Greece

Arthur B. Pardee, PhD
Division of Cell Growth and Regulation
Dana-Farber Cancer Institute
Boston, MA

Ira Pastan, MD
Laboratory of Molecular Biology
National Cancer Institute
Bethesda, MD

William E. Paul, MD
Laboratory of Immunology
National Institute of Allergy and
Infectious Diseases
Bethesda, MD

D. Keith Peters, MB, BCh
School of Clinical Medicine
University of Cambridge
Cambridge, United Kingdom

Lennart Philipson, MD, PhD
Karolinska Institutet
Stockholm, Sweden

Darwin J. Prockop, MD, PhD
Center for Gene Therapy
Allegheny University
Philadelphia, PA

Stanley B. Prusiner, MD
Department of Neurology
University of California, San Francisco
San Francisco, CA

Marlene Rabinovitch, MD
Division of Cardiovascular Research
Hospital for Sick Children
Toronto, Canada

Klaus Rajewsky, MD
Institute of Genetics
Cologne, Germany

Peter G. Schultz, PhD
Department of Chemistry
University of California
Berkeley, CA

Solomon H. Snyder, MD
Department of Neuroscience
Johns Hopkins University School of
Medicine
Baltimore, MD

Donald F. Steiner, MD
Howard Hughes Medical Institute
Chicago, IL

Paul Talalay, MD
Department of Pharmacology & Molecular
Sciences
Johns Hopkins University
Baltimore, MD

Emil R. Unanue, MD
Department of Pathology
Washington University School of Medicine
St. Louis, MO

Inder M. Verma, PhD
Laboratory of Genetics
The Salk Institute for Biological Studies
La Jolla, CA

Denisa D. Wagner, PhD
The Center for Blood Research
Harvard Medical School
Boston, MA

Irving L. Weissman, MD
Department of Pathology and
Developmental Biology
Stanford University School of Medicine
Stanford, CA

Charles Weissmann, MD, PhD
Professor, Institut fur Molekularbiologie I
Universitat Zurich
Zurich, Switzerland

Hans Wigzell, MD, PhD
Karolinska Institutet
Stockholm, Sweden

James M. Wilson, MD, PhD
Institute for Human Gene Therapy
Wistar Institute
Philadelphia, PA

Tadataka Yamada, MD
Department of Internal Medicine
University of Michigan
Ann Arbor, MI

Cover: Ultrathin frozen liver sections from a *spf^{fash}* mouse treated with Ad.mOTC (A), control C3H mouse (B), untreated *spf^{fash}* mouse (C), and *spf^{fash}* mouse treated with Ad.hOTC (D). Labeled with rabbit anti-OTC antibody followed by gold-conjugated goat anti-rabbit antisera. Representative mitochondria are shown from each mouse. See article by Zimmer et al. on page 244 in this issue.

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